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14. ABSTRACT
The **purpose** of this project is to identify predictive markers of heterotopic ossification in an established animal model that would forecast development of heterotopic ossification (HO) in humans soon after injury. Blast procedures have been completed on all 30 animals (Groups I & II) in the year 1 SOW and 45 animals (Groups III – V) in the year 2 SOW. All animals were biopsied and have been sacrificed according to protocol schedule. Groups I and II animals were also followed with scheduled routine radiographs to monitor progression of HO. Specimen samples are under analysis for gene and protein level expression with the Nesti partnering molecular biology lab. In the upcoming year, early-appearing gene and protein biomarkers will be identified by analyzing correlation in animals exhibiting radiographic evidence of HO and will be compared to gene expression signatures in existing human tissue samples known to have gone on to develop HO by the partnering lab.

15. SUBJECT TERMS
Heterotopic ossification, blast injury, amputation, bone formation, animal model, rat model, gene expression, protein expression, biomarkers

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Introduction:

Heterotopic ossification (HO), characterized by the pathologic formation of mature bone in the soft tissues, is a frequent complication following high energy orthopaedic trauma. HO is prevalent in patients with severe extremity war-time wounds; specifically, its incidence is reported between 57-63% in patients that sustain a poly-trauma blast injury [1,2]. Complications related to HO in residual limbs following blast amputation include pain, overlying skin and muscle breakdown, poor fitting and functioning of prosthetic limbs, reoperation for amputation revision, and impaired limb function that delays or limits rehabilitation [3-7]. Current treatments to prevent HO are limited to mitigation rather than prevention. Furthermore, removal of heterotopic bone after it has formed can be difficult; this frequently requires resection of substantial amounts of soft tissue and risks injury to adjacent neurovascular structures that are often intimately associated with the ectopic bone. Hence, it is preferable to address the issue of HO before it begins. Prevention of HO in residual limbs is needed to offer amputation survivors the best possible quality of life and return to function. We have developed a validated blast amputation animal model and confirmed that it replicates the human condition with respect to formation of HO. The current studies are directed at identifying early-appearing biomarkers in the animal model that predict the occurrence of HO in our experimental animals and determine if a correlation exists to similarly predict the development of HO in the human condition. Patients exhibiting biomarkers predictive of exuberant HO formation can then be identified before the disease process begins and treated prophylactically.

Keywords:
Heterotopic ossification, blast injury, amputation, bone formation, animal model, rat model, gene expression, protein expression, biomarkers

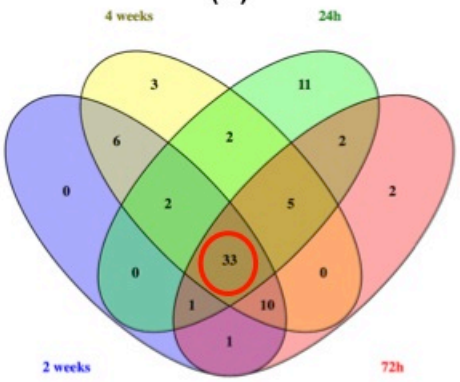
Overall Project Summary:

Current objectives: **All 75 hind-limb blast amputation procedures under Specific Aims 1 & 2 in year 1 & 2 SOW (Groups I – V) have been completed, and all 150 specimens from both amputated and contralateral control limbs have been collected.** Group I and II animals (15 each) were followed with serial radiographs to monitor progression of HO and sacrificed at 24 weeks post-blast, per protocol. Group I animals underwent bilateral muscle biopsy procedure at two weeks, while Group II animals underwent biopsy procedure at four weeks. Group III – V animals (15 each) were biopsied at 24 hours, 72 hours, and 72 hours, respectively, and sacrificed at the same time of biopsy procedures, as per protocol. Group III and IV animals underwent standard wound care with bulb syringe irrigation prior to wound closure following blast amputation while Group V animals underwent pulsed lavage irrigation prior to wound closure. All the biopsy specimens have been sent to the Nesti partner lab for analysis. They were processed to collect total RNAs and protein lysates for identifying both gene- and protein-level biomarkers and will be compared to gene expression signatures in existing human tissue samples known to be characteristic for the formation of heterotopic ossification.

Results: HO progression has been assessed and graded between immediate post-blast and post-mortem radiographs on Group I & II animals. **Radiographic HO data acquired from Group I & II animals are included in supplemental appendix, #1. Biomarker expression data are included in supplemental appendices, #2 & 3.**

Biomarker analysis of animal biopsy specimens was performed by the Nesti partner lab in order to identify molecular predictors of HO. qRT-PCR and Western blot analysis were carried out to examine the expression of fibrosis markers, such as TGF-b1, Col1al, Acta2, Smad3, and fibronectin. Biomarker analysis was performed using the Osteogenesis pathway specific PCR Array (SABiosciences), which contains 84 genes. Different stages of rat biopsy RNA samples (24 hours, 72 hours, 2 weeks, and 4 weeks post-injury) were used for this screening. Data analysis was performed using the RT² Profiler PCR Array Data Analysis software (SABiosciences). For further analysis, the gene lists (fold change greater than 2) from each stage were subjected to a Venn diagram analysis. As a result, 33 genes overlapped among these four stages (Figure 1A). Figure 1B shows the list of 33 genes common to the four stages. From these genes, 10 genes (Ctsk, Fn1, Runx2, Col5a1, Cdh11, Bgn, Bmp1, Col4a1, Mmp2, and Fgf2) showed a significant p-value (at least three different stages) and a gradual increase or decrease in gene expression as the stages developed. Five genes (Fn1, Runx2, Col5a1, Bgn, and Fgf2) all exhibited statistically significant p-values ($P<0.05$), indicating that these genes would be good candidates for HO prognostic markers (Figure 1C).

(A)



(B)

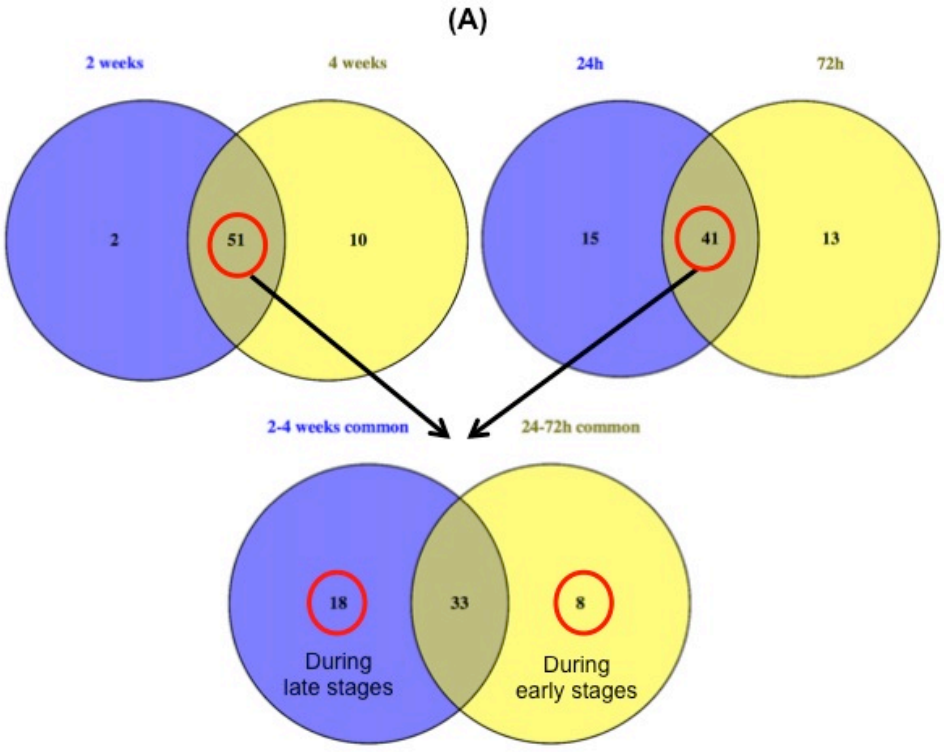
Common	genes	(33)
Anxa5	Fn1	Spp1
Bgn	Gdf10	Tgfb1
Bmp1	Icam1	Tgfbr3
Cdh11	Itga3	Tnf
Chrd	Itgam	Twist1
Col4a1	Mmp2	Actb
Col5a1	Mmp8	Bmpr1b
Csf1	Mmp9	Fgf2
Csf2	Nfkb1	Phex
Ctsk	Runx2	Smad3
Fgfr1	Serpinh1	Hprt1

(C)

	24h		72h		2 weeks		4 weeks	
Gene Symbol	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
Ctsk	2.0542	0.142085	6.4998	0.002901	67.8814	0.000495	79.6499	0.000017
Fn1	9.1137	0.024842	15.2182	0.003014	51.0874	0.000008	52.2679	0.04867
Runx2	10.0585	0.003918	16.3667	0.000003	22.6571	0.00323	35.3669	0.00037
Col5a1	3.4657	0.024589	13.175	0.004965	19.3413	0.028831	34.595	0.011237
Cdh11	2	0.376233	10.972	0.001044	19.5829	0.032844	32.5582	0.000209
Bgn	2.3219	0.01989	10.3869	0.002332	12.6679	0.025057	18.7003	0.002462
Bmp1	5.335	0.082085	7.3709	0.00079	8.2582	0.010878	17.253	0.026136
Col4a1	6.1936	0.011952	6.4163	0.00373	16.0654	0.087917	17.1736	0.004883
Mmp2	3.8063	0.033514	4.5226	0.000507	12.1886	0.088523	16.8669	0.003383
Fgf2	-6.6313	0.002486	-6.1637	0.002686	-4.2115	0.006449	-3.9014	0.006343

Figure 1. Osteogenesis pathway specific PCR array. (A) Venn Diagram analysis. The gene lists were generated from different stages (FC>2) and applied to the Venny website (*Oliveros, J.C. (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams.* http://bioinfogp.cnb.csic.es/tools/venny/index.html.). Thirty-three genes commonly appeared in four different stages. (B) List for common elements (33) genes. (C) Table for common elements genes (10) with fold change and significant p-value (at least from three stages).The significantly altered genes through all different stages were bolded (FC>2, P<0.05).

Venn Diagram analysis was applied to understand the gene signatures specific to only the early (24-72 hours) or late (2-4 week) stages. The commonly expressed genes from the groups were applied to the Venny website to exclude common genes. From this, 18 and 8 genes were identified from only the 4 week and 24hr stages, respectively (Figure 2A). Specifically, 11 genes (B2m, Col4a1, Col6a1, Itga2, Itgav, Rplp1, Tgfb3, Tgfbr1, Tgfbr2, Tnfsf11, and Vcam1) correlated significantly (FC>2, P<0.05) during the 2- 4 week late stage development (Figure 2B). Also, 4 genes (Acvr1, Egf, Smad1, and Tgfb2) correlated significantly (FC>2, P<0.05) during the 24- 72hr early stage development (Figure 2C).



(B)

Gene Symbol	2 weeks Fold Regulation	2 weeks p-value	4 weeks Fold Regulation	4 weeks p-value
B2m	2.0921	0.026578	2.1351	0.014091
Bmp3	9.7624	0.011925	2.7978	0.145456
Col14a1	3.7448	0.01847	17.1736	0.004883
Col1a1	45.791	0.067178	92.9935	0.007174
Col1a2	17.11	0.070204	26.7165	0.015224
Col3a1	12.0057	0.08203	19.943	0.017456
Col6a1	10.6815	0.014757	18.7992	0.003193
Igf1	4.5118	0.063072	7.4764	0.000542
Itga2	2.0928	0.027046	2.702	0.037059
Itgav	3.5705	0.00011	4.0076	0.001922
Mmp10	-8.9524	0.208666	-5.543	0.211671
Rplp1	-2.3075	0.019155	-2.0134	0.028561
Sost	4.2856	0.28621	3.1255	0.322227
Tgfb3	2.4405	0.022365	2.1926	0.046519
Tgfbr1	2.8619	0.000252	2.4859	0.000037
Tgfbr2	4.9922	0.020651	6.1297	0.002315
Tnfsf11	72.1467	0.003664	70.4569	0.000009
Vcam1	7.886	0.000041	8.6159	0.000181

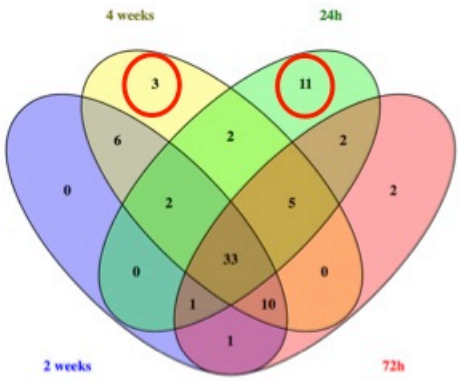
(C)

Gene Symbol	24h Fold Regulation	24h p-value	72h Fold Regulation	72h p-value
Acvr1	-3.4102	0.048519	-2.0226	0.030831
Bglap	2.6242	0.147902	3.4505	0.013198
Bmp5	-2.2522	0.072307	-4.1468	0.137448
Bmp6	-2.329	0.128794	-4.7168	0.000825
Egf	-11.3511	0.000437	-6.6691	0.00107
Ldha	-2.1063	0.103812	-2.8208	0.057299
Smad1	5.446	0.000533	2.4749	0.031182
Tgfb2	-2.999	0.012636	-3.5154	0.023678

Figure 2. Gene signatures during both early and late stages. (A) Venn Diagram analysis. The gene lists commonly expressed in both 2-4 week and 24-72hr stages were applied to the Venny website to exclude common genes. As a result, 18 and 8 genes appeared during the early or late stages. (B) Table for late stage genes with fold change and p-value. (B) Table for early stage genes with fold change and p-value. The significantly altered genes through these two different stages were bolded (FC>2, P<0.05).

The partnering lab has also looked into the gene signature at the early 24h stage and then at the late 4 week stage. If significant genes were missing from the common gene analysis through all stages, gene signatures were observed from as early as the 24 hr stage to as late as the 4 week stage. From common gene analysis using the Venn Diagram (Figure 3A), 3 and 11 genes were from only the 4 week or 24hr stages, respectively. Among three genes, Smad2 was the only significantly altered gene (FC>2, P<0.05) at only 4 weeks (Figure 3B). Three genes (Bmp2, Igf1r, and Sox9) from the 11 genes were the only significant genes (FC>2, P<0.05) from 24hrs (Figure 3C). This gene signature may indicate that this gene also plays a role in the early or late stages of HO progression, temporary. Smad2 is a well- known marker for fibrosis and Bmp2 and Sox9 is also a well- known marker for bone and cartilage. Finally, many of the genes identified from these analyses still need further molecular and cellular biology investigation to better understand their function. All in all, this will result in a greater comprehension of their role in HO progression or as detection markers from our current Rat blast injury model for HO.

(A)



(B)

	4 weeks	
Gene Symbol	Fold Regulation	p-value
Dlx5	2.4377	0.153528
Smad2	2.1208	0.00418
Vegfb	-4.4879	0.142114

(C)

	24h	
Gene Symbol	Fold Regulation	p-value
Alpl	3.8442	0.249303
Bmp2	9.3054	0.02868
Bmp4	3.5313	0.255921
Col10a1	2.0123	0.300441
Csf3	19.0235	0.314614
Flt1	2.6456	0.149989
Igf1r	4.75	0.007464
Itgb1	2.456	0.076968
Nog	2.6867	0.112298
Pdgfa	2.9728	0.093237
Sox9	3.4948	0.032079

Figure 3. Gene signatures from the early (24hr) and late (4 week) stages. (A) Venn Diagram analysis. The red circles indicate genes that are from only the 4 week or 24hr stages. (B) Table for only 4 weeks stage genes with fold change and p-value. (C) Table for only 24h stage genes with fold change and p-value. The significantly altered genes were bolded (FC>2, P<0.05).

Progress and Accomplishments: The project is on schedule as proposed and implemented at our institution. All hind-limb blast amputation procedures on 75 animals have been completed, as well as related scheduled biopsies as specified under Specific Aims 1 & 2. The harvested specimens have been sent to the Nesti partner lab and are currently undergoing RNA profiling using an osteogenesis PCR array to examine the correlation of osteogenic marker expression with radiographic HO findings. Human tissue sample collection from wounded service personnel as specified under Specific Aim 3 will start when the partnering PI obtains IRB approval. Delays were encountered because the partnering lab's USUHS online IRB submission system was changed to a new system resulting in delay of IRB approval. Further delays are anticipated until IRB approval is received. Once the partnering lab has IRB approval, they will be able to collect human tissue samples for analysis and comparison with the animal results.

Key Research Accomplishments: Animal experiments completed on schedule and processing ongoing with data becoming available on a rolling basis as tissue samples are processed and analyzed. Correlation analysis will be performed as data collection from samples is more complete.

Conclusion: Research work is on schedule as proposed and planned. Research conclusions and clinical importance will be determined, as data and analysis are complete. Nothing further to report.

Publications, Abstracts, and Presentations: Nothing to report.

Inventions, Patents and Licenses: Nothing to report.

Reportable Outcomes: Nothing to report.

Other Achievements: The experience and training provided by this award during the prior year directly contributed to the successful hiring of the past research resident to a position in the Orthopaedic residency at the Medical University of South Carolina.

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Appendices:

1. HO radiographic data – Group I & I animals.

Rat #	Biopsy Time	Post-op Radiographic Measurements (mm)		Postmortem Radiographic Measurements (mm)		%L	%W	HO Grade			HO Severity Score
		Length	Width	Length	Width			L	W	Overall	
1	2 weeks	8.85	10.9	11.3	12.1	27.7	11.0	moderate	mild	moderate	2
2	2 weeks	13.7	8.98	15	12.3	9.5	37.0	mild	moderate	moderate	2
3	2 weeks	11.6	16.8	13.1	17.8	12.9	6.0	mild	mild	mild	1
4	2 weeks	12.4	10.2	14.7	10.5	18.5	2.9	mild	mild	mild	1
5	2 weeks	15	6.66	12.7	7.38	-15.3	10.8	mild	mild	mild	1
6	2 weeks	15	11.9	12.6	7.94	-16.0	-33.3	mild	mild	mild	1
7	2 weeks	12.7	9.36	11.9	10.3	-6.3	10.0	mild	mild	mild	1
8	2 weeks	15.8	7.35	19.3	9.04	22.2	23.0	mild	mild	mild	1
9	2 weeks	8.87	10.8	10.9	12.1	22.9	12.0	mild	mild	mild	1
10	2 weeks	9.83	17.5	15.4	13	56.7	-25.7	severe	mild	severe	3
11	2 weeks	8.01	10.6	5.82	8.31	-27.3	-21.6	mild	mild	mild	1
12	2 weeks	9.89	7.64	8.31	9.88	-16.0	29.3	mild	moderate	moderate	2
13	2 weeks	9.36	10.7	14.8	9.82	58.1	-8.2	severe	mild	severe	3
14	2 weeks	14.9	9.04	15.9	8.63	6.7	-4.5	mild	mild	mild	1
15	2 weeks	10.1	10.7	12.7	10.8	25.7	0.9	moderate	mild	moderate	2
16	4 weeks	8.93	8.13	11.4	8.35	27.7	2.7	moderate	mild	moderate	2
17	4 weeks	15.8	11.1	17	11	7.6	-0.9	mild	mild	mild	1
18	4 weeks	11.5	12.6	8.41	12.7	-26.9	0.8	mild	mild	mild	1
19	4 weeks	7.39	8.29	5.81	11.8	-21.4	42.3	mild	moderate	moderate	2
20	4 weeks	13.5	10.3	15.2	11.6	12.6	12.6	mild	mild	mild	1
21	4 weeks	15.1	8.05	15.3	15.3	1.3	90.1	mild	severe	severe	3
22	4 weeks	16.7	8.29	19.7	16.2	18.0	95.4	mild	severe	severe	3
23	4 weeks	13.5	12.8	14.2	11.1	5.2	-13.3	mild	mild	mild	1
24	4 weeks	9.25	14.8	10.7	18.6	15.7	25.7	mild	moderate	moderate	2
25	4 weeks	19.7	10.6	16.8	16.2	-14.7	52.8	mild	severe	severe	3
26	4 weeks	15	7.81	17	9.68	13.3	23.9	mild	mild	mild	1
27	4 weeks	10.1	20	13.3	19.6	31.7	-2.0	moderate	mild	moderate	2
28	4 weeks	9.41	9.09	9.11	9.25	-3.2	1.8	mild	mild	mild	1
29	4 weeks	20	8.26			0.0	0.0	mild	mild	mild	1
30	4 weeks	15.8	7.19	13.9	15.7	-12.0	118.4	mild	severe	severe	3

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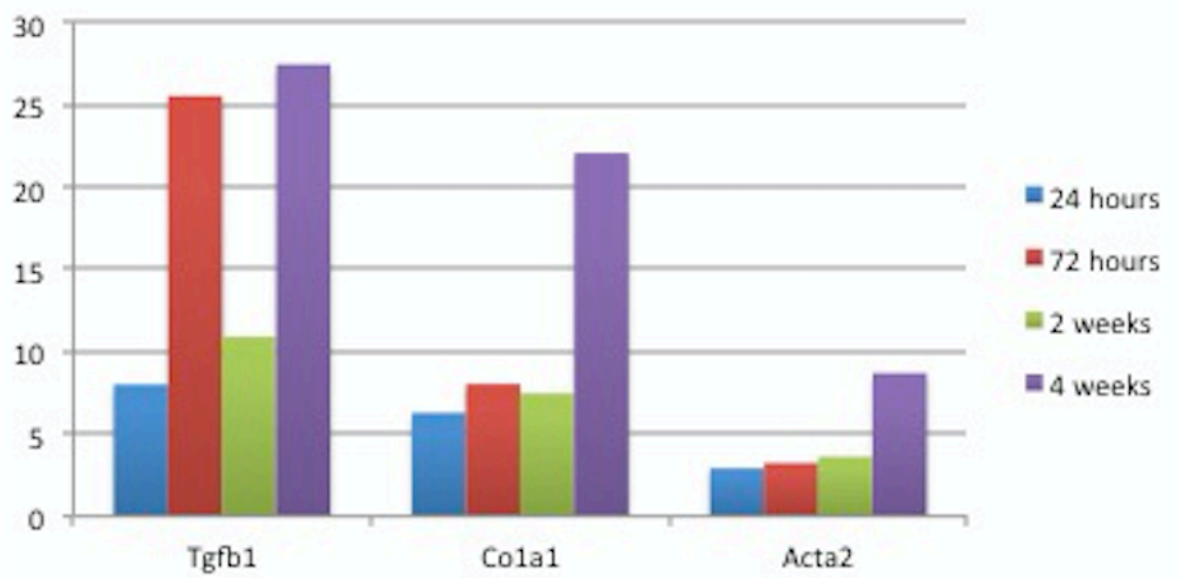
2. Biomarker expression of animal biopsy specimens by qRT-PCR (provided by Nesti partner lab).

Biopsy Time points	Fold Changes		
	Tgfb1	Co1a1	Acta2
24 hr BBS	8.005	6.286	2.917
72 hr BBS	25.533	8.034	3.236
2 week BBS	10.9	7.462	3.622
4 week BBS	27.446	22.058	8.704



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3. Biomarker expression of animal biopsy specimens by western blot (provided by Nesti partner lab).